EXHIBIT B

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A novel diamino-pyridine derivative (IS-741) attenuates rat ileitis induced by trinitrobenzene sulfonic acid

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Background. The etiology and pathogenesis of inflammatory bowel disease remain unknown. However, neutrophil infiltration into the inflammatory lesion is an important process in inflammatory bowel disease. In this study, we used rat trinitrobenzene sulfonic acid (TNBS) ileitis as a Crohn's disease model, and investigated the effects of oral IS-741 (which inhibits the expression of Mac-1, a cell adhesion molecule) on leukocyte-endothelial interactions. ***Methods.*** Rat ileitis was induced by the intraluminal injection of a TNBS solution (160mg/kg in 50% ethanol) at a site 10cm proximal to the ileocecal valve. The rats then received oral IS-741 (50mg/kg) or saline for 7 days. On the day 8 after the initial administration of IS-741 or saline, we determined the visible damage score, and assessed myeloperoxidase (MPO) activity. Concentrations of cytokines in the ileum, such as interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) were assayed by enzyme-linked immunosorbent assay (ELISA). We also investigated the infiltration of polymorphonuclear cells and Mac-1 positive cells by histological examinations. ***Results.*** The administration of IS-741 resulted in a significant reduction of the visible damage score, myeloperoxidase (MPO) activity, and mucosal IL-8 levels in the ileum as compared with the saline administration. IS-741 also dramatically reduced the infiltration of polymorphonuclear cells and Mac-1 positive cells into the inflamed lesions. ***Conclusions.*** These results indicate that the oral administration of IS-741 inhibits neutrophil infiltration into inflamed lesions, and is effective for attenuating rat TNBS ileitis. This new anti-inflammatory agent may be beneficial for the treatment of inflammatory bowel disease.

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Key words: inflammatory bowel disease, Crohn's disease, adhesion molecules, leukocyte-endothelial interaction, trinitrobenzene sulfonic acid

Introduction

The etiology and pathogenesis of inflammatory bowel disease (IBD), such as Crohn's disease and ulcerative colitis, remain quite unknown, and there are no totally curative treatments. However, in patients with ulcerative colitis and Crohn's disease, the infiltration of inflammatory cells, including neutrophils, lymphocytes, monocytes, and macrophages, has been observed in the inflamed tissues. Among these cells, neutrophils play an important role during the early phase of the inflammation. Infiltrating neutrophils release various inflammatory mediators, such as reactive oxygen species (ROS), eicosanoids, and cytokines, that are involved in the pathogenesis of IBD.[1–4] Neutrophils express cell adhesion molecules (CAMs) on their cell surfaces, and are activated, and then they roll, adhere to the vascular endothelial cells, and subsequently infiltrate the inflamed lesion via the CAMs.[5] It has also been reported that cells expressing CAMs are strongly upregulated at the site of inflammation in IBD patients.[6–10] Therefore, the inhibition of neutrophil infiltration into the inflamed lesion is thought to be one of the most effective tools for the treatment of IBD. Clinically, it has been observed that granulocytapheresis and leukocytapheresis, which are methods to remove activated granulocytes and leukocytes, respectively, are effective for attenuating the inflammation of IBD.[11–13]

Recently, it has been demonstrated that treatment with IS-741, a novel N-(2-sulfonylamino-5-trifluoromethyl-3-pyridyl) carboxamide derivative which inhibits the leukocyte-endothelial interaction,[14,15] resulted in a significant reduction of inflammation in

various inflammatory experimental models, as a consequence of its inhibitory effect against neutrophil accumulation.[15–18] However, there are only few reports about the efficacy of leukocyte-endothelial interaction inhibitors against intestinal inflammation.[19]

In the present study, we investigated the effects of oral IS-741 on rat experimental ileitis. A trinitrobenzene sulfonic acid (TNBS) colitis model was reported by Morris et al.,[20] and it has been used as a Crohn's disease colitis model. We modified this TNBS colitis model by the method of intraileal injection of TNBS and have used this experimental ileitis model for the assessment of nutritional therapy,[21,22] because the intraluminal injection of TNBS in ethanol induces T-helper 1 cell (Th1)-mediated transmural inflammation, similar to findings in Crohn's disease.[23,24]

Table 1. Criteria for scoring enteritis

Criteria	Score	
Adhesion	0	None
	1	Minimal
	2	Involving several bowel loops
Strictures	0	None
	2	Mild
	3	Severe, proximal dilatation
Ulcers	0	None
	1	Hyperemia and thickening of intestinal wall
	2	Ulceration and thickening of intestinal wall
Wall thickness	0	Less than 0.5 mm
	2	0.5–1.5 mm
	3	More than 1.5 mm
Maximum score	10	

Materials and methods

Animals

Adult male Sprague-Dawley rats, weighing 300–330g, were obtained from Nippon Clea (Tokyo, Japan) and were acclimatized for 1 week before the experiment. They were housed individually in a room maintained at 22°C, with a 12-h day/night cycle throughout the experiment, and were allowed normal chow (CE-2; Nippon Clea) and water ad libitum. The Animal Care and Use Committee of the Shiga University of Medical Science, Shiga, Japan, approved the study protocol.

TNBS ileitis and IS-741 administration

The rat ileitis in this study was induced according to the method of Tsujikawa et al.,[21,22] with the modifications for rat colitis described by Morris et al.[20] In brief, after starvation of the rats for 36h, a middle abdominal laparotomy was performed with the animals under pentobarbital anesthesia (40mg/kg, i.p.). Then 2,4,6-trinitrobenzene sulfonic acid (TNBS; Tokyo Kasei Kougyou, Tokyo, Japan) was dissolved in 50% ethanol to a concentration of 160mg/ml. The rats were given the TNBS solution (1ml/kg) intraluminally 10cm proximal to the ileocecal valve, with a 29-G syringe. Control rats were given vehicle alone. All rats were then allowed free access to water and chow again. Rats which received the TNBS solution were randomly divided into two groups. The TNBS + IS-741 group (n = 13) received IS-741 (Ishihara Sangyo Kaisha, Shiga Japan) dissolved in distilled water at 50mg/kg, using an 8-cm oral cannula, once per day for 7 days from the day of the ileitis induction. The TNBS + saline group (n = 15) received saline for 7 days. The control group (n = 13) also received the saline, but without ileitis induction.

Assessment of inflammation

On day 8 after the initial administration of IS-741 or saline, ten surviving rats from each group were killed after the withdrawal of blood from the vena cava, and then a 10-cm segment of the ileum, including the inflammation in its middle portion, was removed as a sample. After weighing, the ten samples, we divided them into two groups at random. Six samples from each group were used for the determination of the visible damage score (Table 1), with a slight modification described by Vilaseca et al.,[25] and for the measurement of intestinal myeloperoxidase (MPO) activity and cytokines. The other four samples from each group were subjected to histological examination. Samples from the other surviving rats (n = 3) in each group were used for immunohistochemical staining of Mac-1 (CD11b/CD18) in the inflamed tissue.

MPO activity and cytokine assays

In this study, a modification of the method of Krawisz et al.[26] was used for the measurement of MPO activity. The inflamed ileal mucosa (10cm) was scraped with a glass slide and stored at −80°C until assay. The sample was homogenized for 60s with hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50mM phosphate buffer; pH 6.0; 200mg/ml) on ice. After centrifugation at 12000g for 30min, the sediment was used for the MPO assay and the supernatant was used for the cytokine assay. The sediment was mixed with HTAB buffer (0.5% HTAB in 50mM phosphate buffer; pH 6.0; 200mg/ml), sonicated for 60s, freeze-thawed three times, and then centrifuged at 12000g for 5min. The final supernatant (0.1ml) was mixed with 2.9ml of 50mM phosphate buffer (pH 6.0), containing 0.167mg/ml o-dianisidine hydrochloride and 0.0005%

hydrogen peroxide. The change in absorbance at 460 nm was then measured with a spectrophotometer (UV-3100; Shimadzu, Kyoto, Japan). One unit of MPO activity was defined as the degradation of 1 μmol of peroxide per min at 25°C. From the supernatant of the homogenized sample, the IL-8 (CINC-1) and TNF-α levels were determined using the rat IL-8 (GRO/CINC-1) ELISA system (Amersham Pharmacia Biotech, Tokyo Japan) and the immunoassay Kit Rat TNF-α Ultrasensitive (TFB, Tokyo Japan), respectively.

Histological examination

The sample was fixed in 10% buffered formalin and embedded in paraffin. Slices 3-μm-thick were then stained with hematoxylin and eosin. The histological assessment of the mucosal damage was performed by counting the number of polymorphonuclear cells in the inflamed tissues. The number of polymorphonuclear cells per high-power field was counted in ten separate areas of each slide superior to the muscularis mucosa at sites of the maximal inflammation.[27] The peripheral white blood cells in each rat were counted using an auto-blood cell analyzer.

Immunohistochemistry

We performed immunohistochemical staining of Mac-1 (CD11b/CD18) in the inflamed lesions by the method of Yu et al.[28] Briefly, animals (n = 3 per group) were perfused for 10 min via the left ventricle with 0.01 M phosphate-buffered saline (PBS; pH 7.4) to wash out the blood, and then they were perfused with fixative containing 4% paraformaldehyde (PFA), 0.5% glutaraldehyde (GLU), and 0.2% picric acid (PA) in 0.1 M phosphate buffer (PB; pH 7.4) at 4°C for 10 min. A 10-cm inflamed segment of the ileum was taken out and immersed in postfixative, containing 4% PFA and 0.2% PA in 0.1 M PB at 4°C. The samples were embedded for 6 h in 10% gelatin in 0.1 M PB at 37°C, and immersed for 3 h in 4% PFA in 0.1 M PB at 4°C. Then 20-μm cryostat sections were collected in 0.1 M PBS containing 0.3% Triton X-100 (PBST) for 4 days at 4°C. The sections were incubated with mouse anti-rat Mac-1 (CD11b/CD18) antibody (Immunotech, Marseille, France) diluted 1:1000 in PBST for 4 days at 4°C, and incubated at room temperature (RT) for 2 h in biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:1000 in PBST. The sections were then placed in avidin-biotin perioxidase complex (Elite; Vector Laboratories, Burlingame, CA, USA) diluted in 1:2000 in PBST for 2 h at RT. The immunoreactivity was visualized by incubation with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3.3'-diaminobenzidine (DAB), 1% ammonium nickel sulfate, and 0.0003% H_2O_2 for 30 min at RT. The stained sections were counterstained lightly with 0.1% neutral red.

Statistical analysis

The values for results are presented as means ± SD for each group of animals studied. Differences were evaluated using one-way analysis of variance (ANOVA), followed by a two-tailed Student's t-test. A P value of less than 0.05 was accepted as statistically significant.

Results

Wet weight and visible damage score

Two rats in the TNBS + saline group died of ileus, which had resulted from severe stenosis, but no rats died in the TNBS + IS-741 and the control groups. Like the results in previous experiments,[10,29,30] all of the rats that received the TNBS solution developed clinical symptoms, such as diarrhea and anorexia. However, the body weight changes in the TNBS + saline group and the TNBS + IS-741 group were not significantly different. The wet weight of the ileum increased in both TNBS ileitis groups. The wet weight of the ileum in the TNBS + IS-741 group, however, was significantly reduced as compared with that in the TNBS + saline group (Fig. 1A). Increased wall thickness was observed following the administration of TNBS in both TNBS groups. Although ulceration was observed around the injection site in the TNBS + saline group, ulcer formation was reduced in the TNBS + IS-741 group (Fig. 2). As a result, the visible damage score in the TNBS + IS-741 group was significantly lower than that in the TNBS + saline group (TNBS + saline group, 7.83 ± 0.75 vs TNBS + IS-741 group, 4.16 ± 0.41; $P < 0.001$) (Fig. 1B).

MPO activity and cytokine concentrations

The MPO activity in the ileum was significantly reduced by the administration of IS-741, as compared with the saline group, and was almost the same as that in the control group (Fig. 3). The TNF-α levels were not significantly different among the three groups. In contrast, the IL-8 (CINC-1) levels in the TNBS + IS-741 group were significantly lower than those in the TNBS + saline group, and were nearly the same as those in the control group (Table 2).

Histological examination

The administration of the TNBS solution to the ileum induced transmural inflammation, which consisted of the infiltration of inflammatory cells, such as neutro-

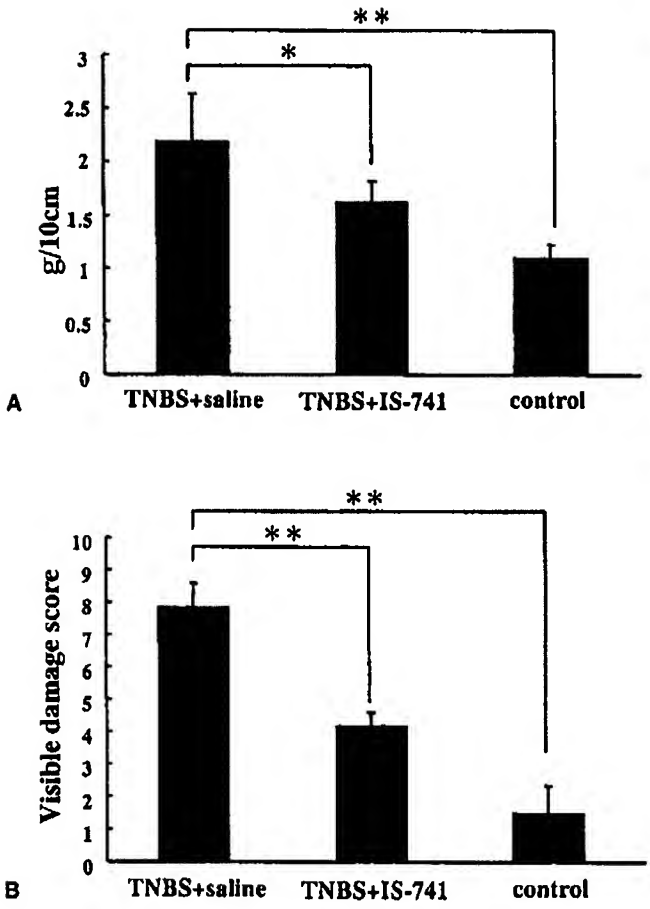

Fig. 1A,B. Effects of IS-741 on (**A**) intestinal wet weight (10-cm segment), and (**B**) visible damage score of the ileum 8 days after the injection of TNBS. Values represent the means ± SD of $n = 6$ animals per group. $^{*}P < 0.05$; $^{**}P < 0.001$. *TNBS*, Trinitrobenzene sulfonic acid

Fig. 2A–C. Macroscopic findings of the ileum 8 days after the injection of TNBS. (**A**) TNBS + saline; (**B**) TNBS + IS-741; (**C**) control. Ulcer and increased wall thickness were observed following the administration of TNBS in the TNBS + saline group. The administration of IS-741 for rat TNBS ileitis reduced the inflammation of the ileum

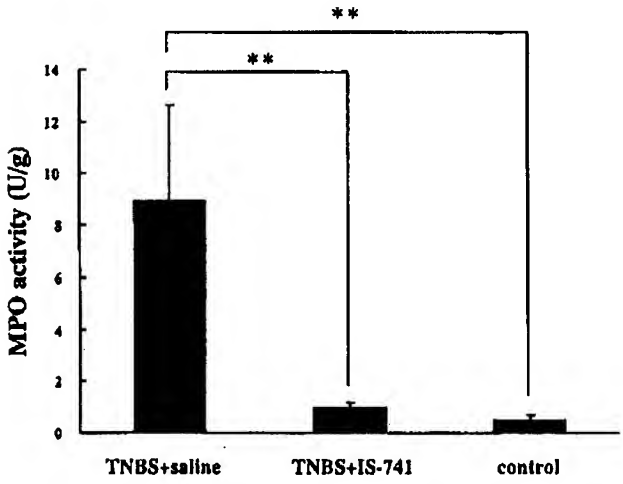

Fig. 3. Effects of IS-741 on myeloperoxidase (*MPO*) activity of the ileum 8 days after the injection of TNBS. Values represent the means ± SD of $n = 6$ animals per group. $^{**}P < 0.001$

phils, lymphocytes, and macrophages. Although massive inflammatory cell infiltration, marked fibrosis of the submucosa, and destruction of the mucosal architecture were noted in the TNBS + saline group (Fig. 4A), inflammatory cell infiltration and fibrosis were dramatically decreased in the TNBS + IS-741 group (Fig. 4B). As shown in Table 3, the number of polymorphonuclear cells in the inflamed lesion of the ileum was significantly decreased in the TNBS + IS-741 group as compared with the TNBS + saline group. The number of white blood cells in each rat increased, but fluctuated in both TNBS groups. There were no significant differences between the IS-741 and saline groups (Table 3).

Immunohistochemistry

Although there were few Mac-1 (CD11b/CD18)-positive cells in the most extensive inflammatory lesions in the TNBS + saline group (data not shown), Mac-1-positive cells accumulated mainly in the inflamed mucosa around the ulcerative lesions (Fig. 5A,A′). In the TNBS + IS-741 group, there were few Mac-1-positive cells either in the most extensive inflammatory lesions or around the inflamed lesions (Fig. 5B,B′).

Discussion

In the present study, the administration of IS-741 for rat TNBS ileitis reduced the infiltration of neutrophils into

Table 2. Mucosal cytokines in the ileum

	TNBS + saline	TNBS + IS-741	Control
IL-8 (pg/ml)	353.1 ± 160.6	90.6 ± 21.3*	99.6 ± 25.6*
TNF-α (pg/ml)	80.6 ± 25.7	85.8 ± 18.6	119.6 ± 39.4

* $P < 0.05$ vs TNBS + saline
Data values are presented as means ± SD ($n = 6$ animals per group). Mucosal interleukin (IL)-8 (CINC-1) levels in the ileum were significantly reduced by IS-741 treatment compared with saline. IS-741 did not affect mucosal tumor necrosis factor (TNF)-α levels in the ileum
TNBS, Trinitrobenzene sulfonic acid

Table 3. Numbers of peripheral WBC and polymorphonuclear cells (PMNS) in the ileum

	TNBS + saline	TNBS + IS-741	Control
Peripheral WBC (/mm³)	5420 ± 2470	6550 ± 2670	4770 ± 1680
PMNS (/high-power field)	26.7 ± 8.4	9.6 ± 6.6*	3.0 ± 0.7**

* $P < 0.05$; ** $P < 0.01$ vs TNBS + saline
Data values are presented as means ± SD ($n = 6$ animals per group). There were no significant differences between peripheral WBC counts in the TNBS + IS-741 group and the TNBS + saline group. The number of PNMS in the ileum was significantly decreased in the TNBS + IS-741 group compared with the TNBS + saline group

the ileum, as shown by the histological examination and by the reduced MPO activity. IS-741 also decreased the mucosal levels of IL-8 (CINC-1), a cytokine that enhances neutrophil chemotactic activity.[31,32]

As we showed in the results, IS-741 attenuated the inflammation of TNBS ileitis. IS-741, a novel diaminopyridine derivative, has been reported to inhibit the adhesion of human promyelo-leukemia cells to human umbilical vein endothelial cells during lipopolysaccharide stimulation in vitro.[14] Although the exact mechanism responsible for these inhibitory effects is unclear, IS-741 is likely to affect adhesion molecules which belong to the β1 or β2 integrin family on the surfaces of the neutrophils, based on antibody experiments.[14] There have been several studies suggesting that this new anti-inflammatory agent was effective for in-vivo animal inflammatory models. Yotsuya et al.[16] reported that IS-741 (0.03 or 0.3 mg/kg per h), administered subcutaneously in a rat severe acute pancreatitis model (induced by trypsin and taurocholic acid) could inhibit the development of the pancreatic lesion as well as the progression to multiple organ failure. Liang et al.[17] reported that intravenous IS-741 (0.03 mg/kg per h) administration for 30 min effectively prevented cerulein-induced pancreatitis and the associated lung injury following an endotoxin challenge. In both these models, the neutrophil infiltration into the inflamed tissues was significantly reduced by the administration of IS-741. It has also been reported that this new agent decreased the number of adherent leukocytes along pancreatic collecting venules, as well as reducing the expression of Mac-1 (CD11b/CD18) on circulating neutrophils, in an acute pancreatitis model,[15] and it also decreased the number of neutrophils and Mac-1-positive cells accumulated in the injured lung.[17] Based on these results, it appears that the leukocyte accumulation during the early phase of pancreatitis and lung injury may be mediated by leukocyte-endothelial cell interactions via the leukocyte integrin Mac-1, and that this new anti-inflammatory agent attenuated the leukocyte-endothelial interactions as a consequence of its inhibitory effect on Mac-1 expression in the inflamed lesions. In this study, we also showed that the infiltration of polymorphonuclear cells and Mac-1-positive cells into the inflamed tissue was dramatically inhibited by the administration of IS-741. Therefore, IS-741 was thought to prevent the ileitis by attenuating the leukocyte-endothelial interactions, similar to the actions thought to occur in the acute pancreatitis model.

Recently, Yotsuya et al.[19] reported that the administration of IS-741 (1, 10, or 100 mg/kg) led to the attenuation of dextran sodium sulfate (DSS)-induced colitis, which is useful for the evaluation of therapeutic effects against ulcerative colitis. They showed that IS-741 decreased the area of erosion in the large intestine and decreased the thickening of the large intestinal wall, as well as attenuating anemia, due to its inhibitory effects on the inflammatory cell infiltration into the intestinal wall. Although several inflammatory mediators, such as IL-1 and IL-8, are involved in both the DSS colitis and the TNBS ileitis model,[33–35] there are some differences between the two models, such as their histological findings, and the distribution of the inflammation. From the results of the present study, we wish to emphasize

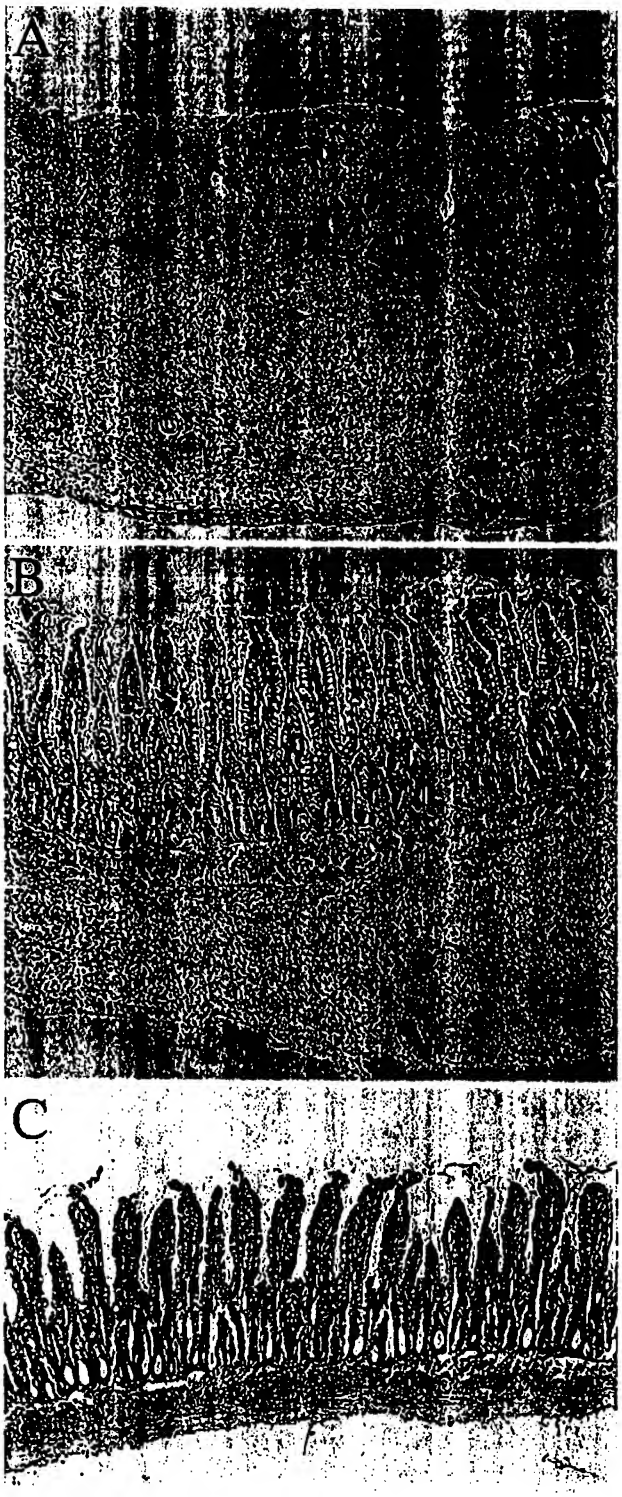

Fig. 4. Histopathological observations of the ileum in (A) TNBS + saline group, (B) TNBS + IS-741 group, and (C) control group. The administration of IS-741 dramatically reduced the inflammatory cell infiltration and fibrosis induced by TNBS. A–C H&E, ×40

that the effectiveness of IS-741 is not limited to DSS colitis, and that IS-741 is also effective in this TNBS ileitis model. Furthermore, for the first time, we elucidated the new mechanism of the therapeutic effect of this agent on intestinal inflammation, such as the effect of IS-741 on the TNF-α and IL-8 levels and MPO activities of the intestine.

IS-741 reduced the tissue damage, as assessed by the visible damage score or by tissue wet weight, by about half, compared with findings in the saline group, whereas IS-741 completely inhibited the function of the neutrophils in the inflamed lesion, based on the results of the MPO activity and IL-8 (CINC-1) concentration experiments. IS-741 was administered orally once per day for 7 days at a dose of 50 mg/kg. It has been reported that the mucosal inflammation induced by TNBS reached its maximum 7 days after the injection, and that oral IS-741 was rapidly absorbed.[21] Thus, we believe that the dose, administration route, and duration of IS-741 administration are adequate for this model. Therefore, the reason for the differences between the MPO activity or IL-8 concentration and the visible damage score may be that this new anti-inflammatory agent has effects mainly on neutrophils, and that it has little effect on other inflammatory cells, such as lymphocytes and epithelial cells, which are also associated with the process of this experimental ileitis.

It has been reported that IL-8 and TNF-α were the major mediators in the inflammatory process and that the production of these cytokines was enhanced in inflamed intestine of human IBD.[36-42] IL-8, which has potent neutrophil chemotactic activity, is known to persist in its active form for long periods in inflamed tissues.[37,38,43] Indeed, in this study, we showed that the mucosal IL-8 levels were increased in the TNBS + saline group. Interestingly, however, the mucosal TNF-α levels were not increased in the TNBS + saline group. Tateishi et al.[44] examined the time course of tissue levels of TNF-α in the formation of TNBS colitis, and found that the levels of tissue TNF-α began to increase just after the induction of colitis, reached a peak at 45 min, then rapidly returned to near the basal level. We therefore consider that the level of mucosal TNF-α did not show a significant increase probably due to this specific time course of TNF-α, because we measured mucosal TNF-α levels on day 8 after the administration of TNBS.

We expect a trial for the therapeutic efficacy of this new anti-inflammatory agent in human IBD. TNBS ileitis shows transmural inflammation similar to that in Crohn's disease, but the inflammation does not continue for more than 2 weeks. There are still many substantial differences between the experimental model and the human disease. Palmen et al.[45] reported that the rectal administration of dexamethasone, which is effec-

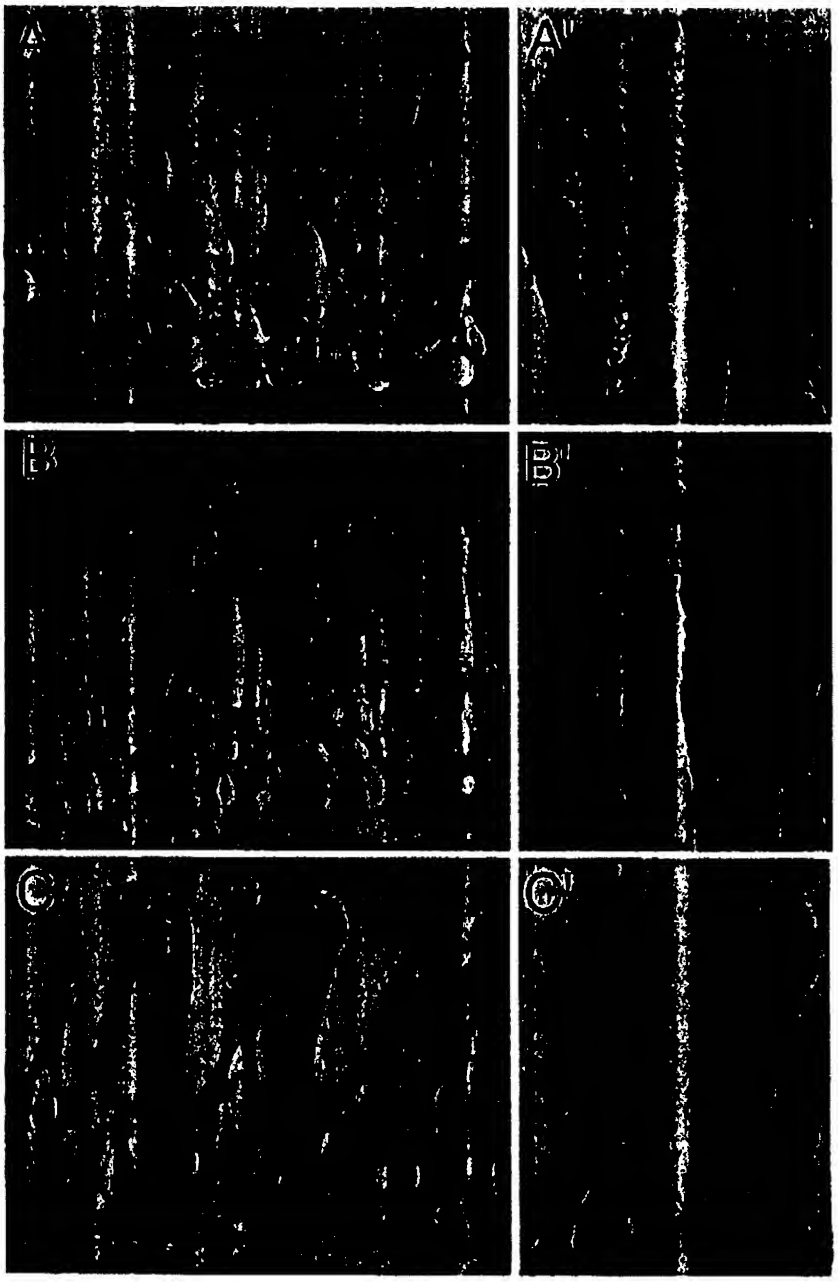

Fig. 5A–C′. Immunohistochemistry for Mac-1 (CD11b/CD18) in the ileum. **(A)** TNBS + saline; **(B)** TNBS + IS-741; **(C)** control; **A′**, **B′**, **C′** higher magnifications of **A**, **B**, and **C**, respectively. Mac-1-positive cells accumulated mainly in the inflamed mucosa around the ulcerative lesions in the TNBS + saline group. The administration of IS-741 dramatically reduced the expression of Mac-1. **A–C** Mac-1 staining, ×100; **A′–C′** Mac-1 staining, ×200

tive for treating human ulcerative colitis, did not attenuate TNBS colitis. Therefore, caution is necessary in extrapolating the experimental results to human patients.

In conclusion, the oral administration of the novel carboxamide derivative IS-741 attenuate rat TNBS ileitis by inhibiting neutrophil infiltration into the inflamed lesions, as a consequence of its inhibitory effect on Mac-1 expression. The clinical utility of this anti-inflammatory agent for human IBD warrants investigation.

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